

Prolactin Suppression of Gonadotropin-Releasing Hormone Initiation of Mammary Gland Involution in Female Rats

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It has been demonstrated that mammary gland involution after lactation is initiated by accumulation of milk in alveoli after weaning. Here, we report that involution is also dependent on mammary GnRH expression that is suppressed by PRL during lactation. Reduction of plasma prolactin (PRL) by the withdrawal of suckling stimuli increased GnRH and annexin A5 (ANXA5) expression in the mammary tissues after lactation with augmentation of epithelial apoptosis. Intramammary injection of a GnRH antagonist suppressed ANXA5 expression and apoptosis of epithelial cells after forcible weaning at midlactation, whereas local administration of GnRH agonist (GnRHa) caused apoptosis of epithelial cells with ANXA5 augmentation in lactating rats. The latter treatment also decreased mammary weight, milk production, and casein accumulation. Mammary mast cells were strongly immunopositive for GnRH and the number increased in the mammary tissues after weaning. GnRHa was shown to be a chemoattractant for mast cells by mammary local administration of GnRHa and Boyden chamber assay. PRL suppressed the mammary expression of both ANXA5 and GnRH mRNA. It also decreased mast cell numbers in the gland after lactation. These results are the first to demonstrate that GnRH, synthesized locally in the mammary tissues, is required for mammary involution after lactation. GnRH is also suggested to introduce mast cells into the regressing mammary gland and would be in favor of tissue remodeling. The suppression of these processes by PRL is a novel physiological function of PRL. (*Endocrinology* 157: 2750–2758, 2016)

The mammary gland develops during pregnancy and lactation under the influence of a variety of hormones, including prolactin (PRL) (1). After parturition, the major function of PRL is milk production and maintenance of an active gland. PRL for this purpose is released through a neuroendocrine reflex to suckling stimuli (2). The gland regresses to a pre-pregnant state shortly after weaning. During this process of postlactational involution, the mammary tissue shrinks to less than half of its weight within a few days. Involution consists of extensive apoptosis of epithelial cells and tissue remodeling. Lack of

milk removal from alveoli due to the cessation of suckling has been suggested to induce involution through the altered local synthesis of the cytokines, leukemia inhibitory factor (LIF) and transforming growth factor β 3 (3–5). These cytokines activate the signal transducer and activator of transcription 3 (Stat3) pathway. Transition from a prolactational activation of Stat5 by PRL to activation of Stat3 by LIF and transforming growth factor β 3 is of primary importance for the first phase of involution (6). Despite knowledge of these intracellular mediators, it is still unclear whether involution occurs entirely as an autono-

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Abbreviations: ANXA5, annexin A5; GnRHa, GnRH agonist; LIF, leukemia inhibitory factor; PRL, prolactin; Stat3, signal transducer and activator of transcription 3; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

mous response of the epithelial cells to milk accumulation or is also caused by changes in the endocrine milieu after weaning, that is cessation of massive PRL secretion.

GnRH is known primarily as a hypothalamic neuro-hormone that regulates reproduction in both sexes through pituitary gonadotropin secretion. Expression of GnRH and its receptor have also been found in various peripheral tissues (7, 8). GnRH suppresses the growth of a variety of tumors through its receptor (7, 9); however, it is difficult to evaluate GnRH action in cells that do not synthesize gonadotropin proteins. We have found previously that annexin A5 (ANXA5) is expressed in pituitary gonadotropes (10), is increased by GnRH stimulation (11, 12) and is involved in GnRH stimulation of gonadotropin secretion (12). ANXA5 is a member of annexin family of proteins grouped by their structural similarity and affinity to calcium and phospholipids (13, 14). ANXA5 is suggested to be involved in the signal transduction of the GnRH receptor (11). We have also examined the relationship between GnRH and ANXA5 in various tissues and found that GnRH stimulates ANXA5 expression in many peripheral tissues, including corpus luteum and Leydig cells (15, 16). Although the relevance of this phenomenon to any physiological function of this protein is not clear, ANXA5 can be used at least as a useful biomarker of GnRH action, especially in peripheral tissues. Similarly, ANXA5 is widely used as a tool to detect early apoptotic cells, by affinity to phosphatidylserine exposed on the outer layer of the plasma membrane (17), whereas its physiological function in this circumstance is still unclear. We previously found that the expression of ANXA5 is dramatically increased in the mammary epithelial cells after weaning (18). This observation strongly suggested a GnRH action in regressing mammary tissues.

It has been reported that mast cells increase in the regressing mammary gland (19–21). Mast cells play a role in anaphylaxis and IgE-mediated allergic disorders as effector cells (22). They are also tissue-resident immune cells involved in both the innate and adaptive immune response. Mast cells differentiate from multipotent bone marrow progenitor cells into at least 2 differentiated cells, namely connective tissue type and mucosal tissue type mast cells (23). Although the role of mast cells in various pathophysiological responses is well characterized, our understanding of the physiological significance of the accumulation of mast cells in regressing mammary tissues is limited (20). Because mast cells contain GnRH (24, 25), we postulate a GnRH-related function during mammary involution.

In the present study, we examined the hypothesis that GnRH is involved in mammary tissue involution and re-

modeling after weaning and that PRL suppresses this process during lactation.

Materials and Methods

Animals

Adult female Wistar Imamichi rats bred in our laboratory showing regular 4-day estrous cycles were used. Mating was performed by placing a proestrous rat in the cage of a male rat. Sperm was confirmed in the vaginal smear in the morning of estrus. The day of delivery was designated as day 1 of lactation. The number of pups was adjusted to 8 on day 2 of lactation. Experimental procedures followed the guidelines of and were approved by the Animal use committee at Kitasato University.

Local administration of GnRH antagonist or agonist to the mammary tissue

GnRH antagonist (Cetrorelix; kindly donated by Zentaris GmbH) or GnRH agonist (GnRHa) (Des-Gly10 [Pro9]-GnRH ethylamide; Takeda Pharm) was administered locally to a hemilateral inguinal mammary gland. The solutions were emulsified with equal volume of sesame oil just before the injection to minimize the diffusion. Emulsified saline was given to contralateral mammary gland for a control. Tissues were subjected to histological examinations, terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) analysis and RNA extraction. Cetrorelix (200 ng/50 μ L) was given to one side of inguinal mammary tissues of the rats forcibly weaned on day 10 of lactation for 2 days. The mammary glands of both sides were collected on day 12. GnRHa (200 ng/50 μ L) was administered to the lateral inguinal mammary gland once a day from day 10 to day 12 of lactation. The mammary glands of both sides were collected on day 12 and subjected to the same experiments as those with Cetrorelix.

Tissue preparation for histological analysis

Rats deeply anesthetized with Somnopentil (50 mg/kg; Kyoritsu Seiyaku Co) were infused with 50 mL of PBS (0.1 M; pH 7.4) via the left ventricle and then with 50 mL of 4% paraformaldehyde. The right atrial auricle was cut off before the infusion. The inguinal mammary glands were collected and were further fixed in 4% paraformaldehyde overnight at 4°C. Tissue blocks were washed, dehydrated, and embedded in paraffin per standard procedure. Four-micrometer tissue sections were made, dried in an incubator at 37°C overnight, and subjected to immunohistochemistry, toluidine blue staining, or TUNEL analysis.

Immunohistochemistry of ANXA5 and GnRH for the mammary tissues were performed with specific antibodies made by us previously (26, 27). Specimens were counterstained with hematoxylin. Apoptotic cells were detected by TUNEL reaction using In Situ Cell Death Detection kit POD (Roche Diagnostics) according to manufacturer's protocol. The sections were counterstained with hematoxylin. Tissue mast cells were detected by metachromatic staining with toluidine blue. For each experiments, at least 2 animals were used, and 3 sections per animal were made.

After xylene treatment and hydration via an ethanol series, the sections were immersed in 1% H₂O₂ for 30 minutes to ex-

haust endogenous peroxidase activity and were blocked with 2.5% normal horse serum for 30 minutes to reduce nonspecific antibody binding. The first antiserum was used at 1:10 000 and the sections were incubated with the serum overnight at 4°C. The second antibody system for visualization used was the Immpress reagent antirabbit IgG POD kit (Vector Laboratories). The specimens were counterstained with hematoxylin. Negative controls were prepared by using normal rabbit serum instead of each first antibody.

Mast cell counting

Mammary tissue sections were stained with 0.1% toluidine blue dye. The number of mast cells in the mammary tissue was counted under a microscope ($\times 20$) for 20 different fields in each of 16 slides from 4 rats each for each experimental group. The number of mast cells was expressed per square millimeter.

Mast cell culture

Adult female rats were killed by decapitation. Mast cells were collected by rinsing the peritoneal cavity with 1% BSA-PBS solution. The cell suspension was centrifuged at 100g for 10 minutes. Pelleted cells were suspended in 10-mL culture medium (α -MEM, [Invitrogen Corp], 10% fetal bovine serum [Sigma-Aldrich Corp], and 1% penicillin 100 U/mL and streptomycin 100 μ g/mL [Invitrogen Corp]), was layered on the top of 10-mL 75% Percoll solution (GE Healthcare Bio-Sciences AB) in 1 \times Hanks' balanced salt solution (Invitrogen Corp). This was centrifuged at 600g for 20 minutes. The bottom 2-mL portion containing mast cells was collected. Mast cells were washed with culture medium (centrifugation at 300g for 5 min) and then cultured at 37°C, 5% CO₂ in a humidified atmosphere.

Chemotaxis assay

Peritoneal mast cells (5×10^4 cells/100 μ L) were placed in the insert of 24-well plates, Transwell Plate (Corning, Inc). Boyden chamber assays for evaluating chemotaxis by measuring cell migration across the polycarbonate membrane with 8- μ m pores within 1 hour were then performed. After 1 hour of incubation, inserts were removed, washed in PBS, and fixed with 10% formalin. The membrane was stained with toluidine blue and cells appearing beneath the membrane were counted.

Real-time PCR

Inguinal mammary tissues were collected from lactating and weaned rats. They were also taken from dams given GnRH α , Cetrorelix, and PRL. Cetrorelix, 200 ng/50 μ L/d, or saline was administered once a day for 2 days locally to different sides of inguinal mammary tissues of the same lactating rat after pup removal on day 10. GnRH α (200 ng/50 μ L/d) or saline was similarly given locally to a lateral inguinal mammary gland of lactating rats from day 10 to 12 of lactation. On day 10 of lactation, pups were removed and PRL was administered sc every 8 hours for 2 days (30 IU/200- μ L 50% of polyvinyl pyrrolidone). Inguinal mammary tissues were dissected and total RNA was extracted with TRIzol reagent (Invitrogen). RNA samples were dissolved in diethylpyrocarbonate-treated water to 500 ng/ μ L and subjected to reverse transcription. RNA was reverse transcribed to cDNA with a High Capacity cDNA Archive kit (Applied Biosystems) according to the protocol supplied by the manufacturer. Then, cDNA samples were subjected to real-time PCR

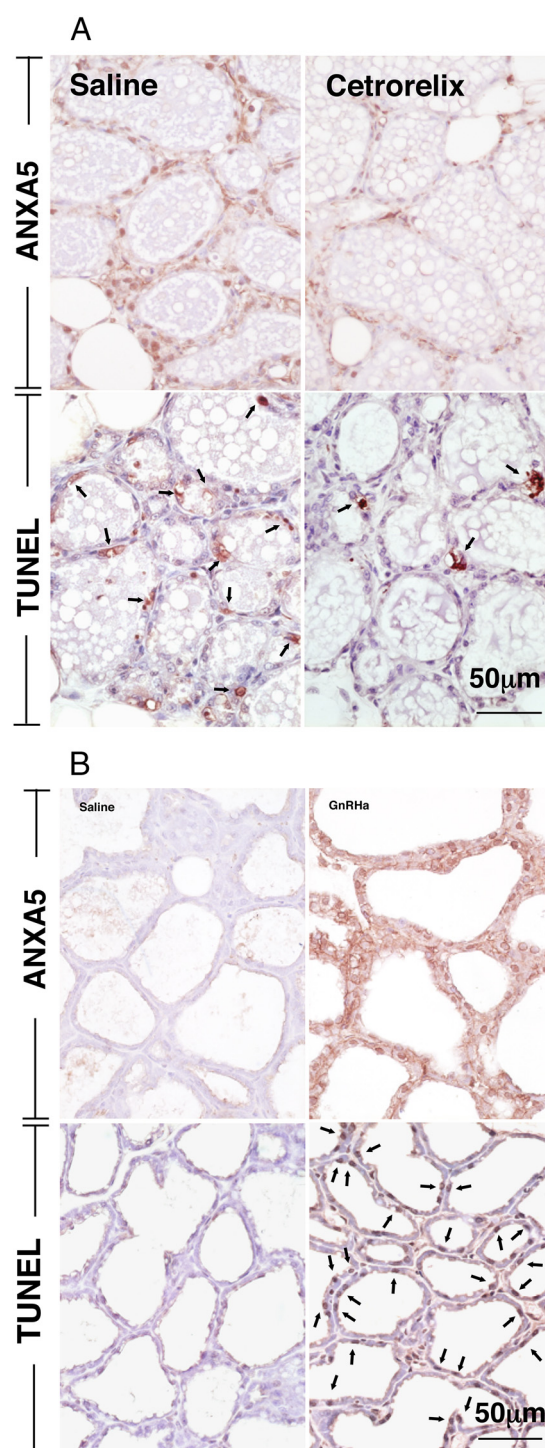


Figure 1. GnRH involvement in ANXA5 expression and apoptosis of mammary epithelial cells after forced weaning at mid lactation. A, GnRH antagonist (Cetrorelix) or saline was administered locally to different side of inguinal mammary tissues for 2 days after pup removal on day 10 of lactation. Mammary tissues were collected on day 12 and subjected to the immunohistochemistry for ANXA5 or TUNEL reaction. B, GnRH α or saline was given locally to the hemilateral inguinal mammary tissues of lactating rats from day 10 for 2 days. Both sides of inguinal mammary gland were subjected to the immunohistochemistry for ANXA5 and TUNEL reaction. Arrows indicate TUNEL-positive reaction. At least 2 rats were prepared for each experiment, and 3 sections per rats were examined. Representative images are shown.

for measurement of the expression of ANXA5 and GnRH mRNA. The internal standard used was TaqMan Ribosomal RNA Control reagent (Applied Biosystems). The ANXA5 primers used were 5'-ATGGCTCTCAGAGGCACCGT-3' for sense primer and 5'-CGTGTTCAGCTCGTAGGCG-3' for antisense primer (based on NM_013132.1). The GnRH primers used were 5'-AGCACTGGTCCTATGGGTTG-3' for sense primer and 5'-TCTGCCATTTGATCCTCCTC-3' for antisense primer (based on NM_012767.2). Power SYBR Green PCR Master Mix (Applied Biosystems) was used for real-time PCR. PCR was performed with 50 cycles of 94°C for 0.5 minutes, 65°C for 0.5 minutes, and 72°C for 1.5 minutes. A series of serially diluted cDNA of mammary tissue was used for drawing a standard curve. Measure was divided by that of RNA control and revealed as expression level. Each experimental group consists of 4 rats.

GnRH assay

Tissue content of GnRH was measured by time-resolved immunofluorometric assay (DELFA). Mammary tissues were obtained on days 20, 23, 26, and 29 after parturition ($n = 4$). They were homogenized in 1M acetic acid, and the homogenates were centrifuged at 15 000g for 15 minutes at 4°C. The supernatant was subjected to GnRH assay after boiling at 100°C for 5 minutes. GnRH was labeled with Europium using DELFIA Eu-labeling kit (PerkinElmer, Inc). Immunoplate (96 wells; Nunc) was coated with 5 μ g/well of antirabbit IgG goat γ -globulin (homemade), and then optimally diluted antirabbit GnRH serum was overlaid. Sample and Eu-labeled hormone were incubated overnight at 4°C, and the intensity of the bound label was measured by ARVO multilabel plate reader (PerkinElmer, Inc.).

Evaluation of milk production

GnRHa (300 ng/600 μ L) or the same volume of saline was given directly to the mammary gland as 50 μ L each to 12 places

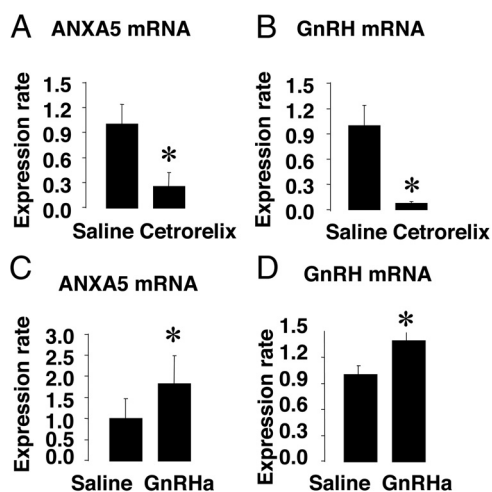


Figure 2. The effects of GnRH antagonist and agonist on ANXA5 and GnRH mRNA expression. A and B, Saline or GnRH antagonist (Cetorelix) was locally administered to the hemilateral inguinal mammary gland for 2 days after pups removal on day 10 of lactation. RNA samples were prepared from mammary tissues on day 12 and subjected to quantitative real-time PCR for ANXA5 and GnRH mRNA ($n = 4$). C and D, Saline or GnRHa was given locally to the hemilateral inguinal mammary gland from lactation day 10 to 12. Mammary expression of ANXA5 and GnRH mRNA expression were determined ($n = 4$). Asterisks reveal significance difference between saline control ($P < .05$).

near every nipple at the time of pup removal on lactation day 10. One group of dams was given the same amount of GnRHa sc on the back. The weight gain of pups (28 pups per 4 dams for each group) after 1 hour of suckling after an 8-hour fast was measured.

Western blotting

The effect of GnRHa on milk protein synthesis was examined by Western blotting for β -casein. Equal amounts (75 μ g) of proteins from each extract were boiled for 5 minutes in sodium dodecyl sulfate sample buffer under reducing conditions, electrophoresed on 12% SDS-PAGE gels, and transferred onto polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membranes were blocked with 1 \times blocking solution for 1 hour at room temperature and then incubated with primary antibody (anti- β -casein or anti- β -actin) at 4°C for 12 hours. After incubation, the membranes were washed 3 times in 0.1% Tween 20-PBS, incubated with peroxidase-labeled antirabbit antibody or antimouse antibody at room temperature for 2 hours, and washed 3 times in 0.1% Tween 20-PBS. Bands were detected by chemiluminescence using ECL Plus Western Blotting (GE Healthcare) according to the manufacturer's instructions.

Statistics

The results are expressed as mean \pm SEM. Statistical analysis was performed using Student's t test for comparison of 2 means and Tukey's test for multiple comparisons. $P < .05$ was considered to be statistically significant.

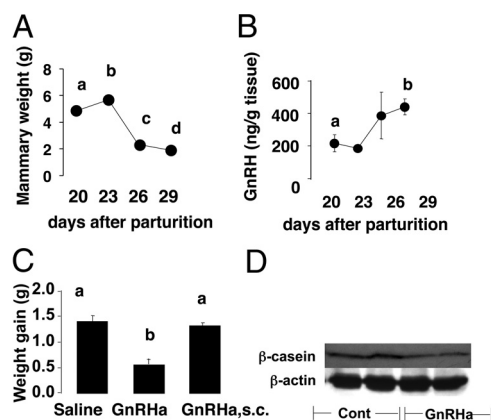


Figure 3. Changes in mammary GnRH content after weaning and the effect of GnRH on milk production. A, Changes in the weight of inguinal mammary tissues after pup removal on day 21 of lactation. Hemilateral inguinal mammary tissues were harvested and weighed from day 20 to 29 ($n = 4$). B, Changes in GnRH content of mammary tissues. Pups were removed on day 21 of lactation. Mammary tissues were collected on each day and the extract was subjected to GnRH measurement ($n = 4$). C, The effect of GnRHa on milk production was evaluated by weight gain of pups after fasting. Pups were removed on lactation day 10, and GnRHa or saline was given locally to the mammary tissues or sc at back. Pups were returned to each dam 8 hours later. Weight gain of pups after 1-hour suckling was measured. Data are expressed as g weight gain. Groups marked with different letters are statistically different, $P < .05$. D, The effect of local administration of GnRHa on mammary milk protein (β -casein). Western blot analysis of β -casein expression. GnRHa was given locally to the mammary tissues on lactation day 10. Mammary tissues were obtained 2 days after. SDS-PAGE, and Western blotting for β -casein and β -actin were performed.

Results

GnRH is involved in mammary involution after lactation

An increase in epithelial cell apoptosis after weaning has been well documented. We have confirmed this in our own work and additionally shown that this coincides with an increase in the expression of ANXA5 both after normal weaning and also 2 days after pup removal at midlactation day 10 (18). The increase in ANXA5 staining and the number of TUNEL-positive cells after pup removal were both suppressed by the local administration of the GnRH antagonist Cetrorelix (Figure 1A). On the other hand, the administration of a GnRH α directly to the inguinal mam-

mary gland of lactating rats increased ANXA5 staining intensity and the number of TUNEL-positive cells even if pups were remained (Figure 1B). Representative images of multiple results are shown.

The effect of Cetrorelix and GnRH α on the expression of ANXA5 and GnRH mRNA expression were examined in forcibly weaned and lactating rats ($n = 4$, respectively). Cetrorelix suppressed both ANXA5 and GnRH mRNA expression in the mammary tissues after forcible weaning (Figure 2, A and B), whereas GnRH α increased both ANXA5 and GnRH mRNA expression even in lactating rats (Figure 2, C and D).

Mammary tissues decreased in weight after weaning on day 21, and the tissue content of GnRH was increased inversely ($n = 4$) (Figure 3, A and B). More direct evidence of GnRH action on the epithelial cells was obtained by measuring tissue weight and milk production. Local administration of GnRH α (200 ng/50 μ L) to the inguinal mammary gland significantly reduced the weight of inguinal mammary tissues after 2 days (7.32 ± 0.19 vs 6.33 ± 0.21 g, $P < .005$). Furthermore, to test the effect of GnRH on milk production, GnRH α (300 ng/600 μ L) was injected directly into the mammary tissue (50 μ L close to all 12 nipples) at the time of pup removal, the pups were fasted for 8 hours and then returned to the dam to suckle for 1 hour. The weight gain of pups during the 1-hour suckling, due to ingestion of milk, was suppressed significantly compared with pups from dams administered saline locally to the mammary tissues (Figure 3C). The weight gain of pups nursed by a dam given the same amount of GnRH α sc to the back was not different from that of controls given saline. The GnRH α was also shown to reduce the mammary content of β -casein (Figure 3D).

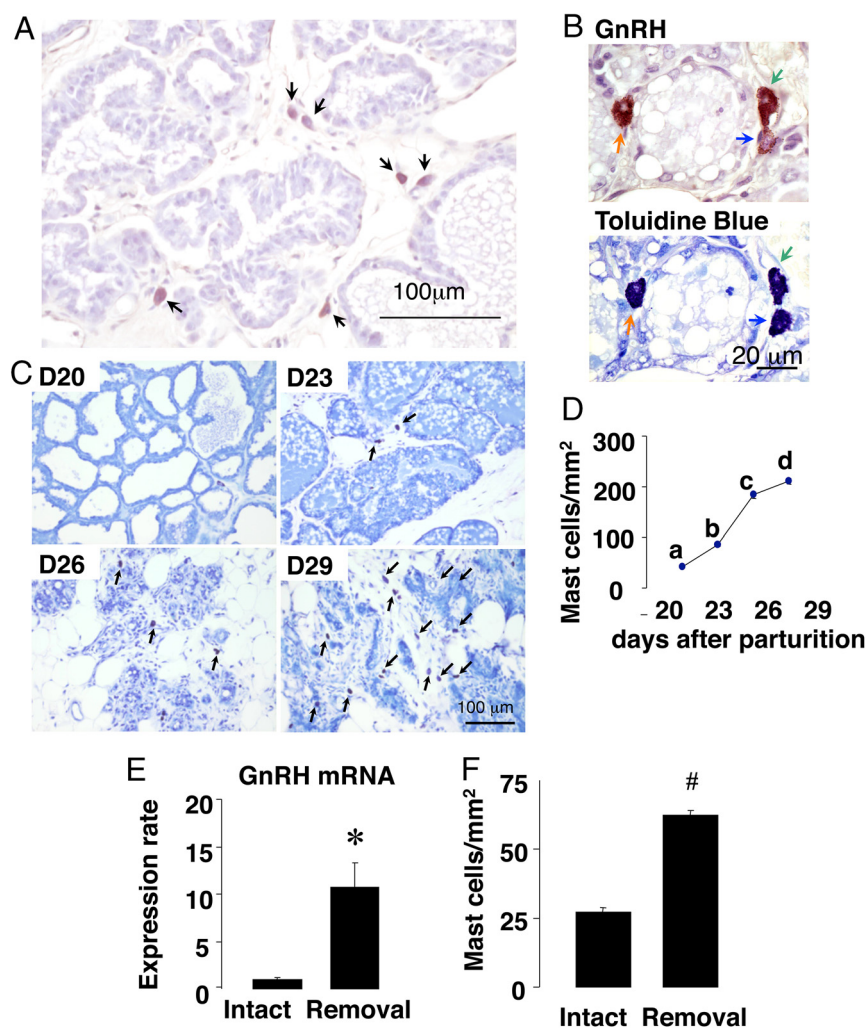


Figure 4. Mast cell expression of GnRH in the mammary tissues and changes in number of mast cells after pup removal. A, Immunohistochemical demonstration of GnRH-positive cells. Arrowheads indicate heavily positive cells. B, Adjacent sections were stained with anti-GnRH and toluidine blue. Arrowheads with the same color indicate same cells. C, Mammary tissues were stained with toluidine blue on day 20, 23, 28, and 29 of lactation. Pups were removed from their mother on day 21. Arrows show mast cells distinguished with metachromasy. D, Changes in mammary mast cell number after weaning on day 21. Groups marked with different letters are statistically different, $P < .05$. E and F, Pups were removed on day 10 of lactation and mammary tissues were collected 2 days after. Mast cells were counted after forced weaning on day 10 of lactation. GnRH mRNA expression was also examined ($n = 4$); *, $P < .05$; #, $P < .001$.

Tissue mast cells express GnRH in the mammary gland and increase during involution

GnRH was shown to localize intensely to large oval cells situated in interlobular tissues of the mammary

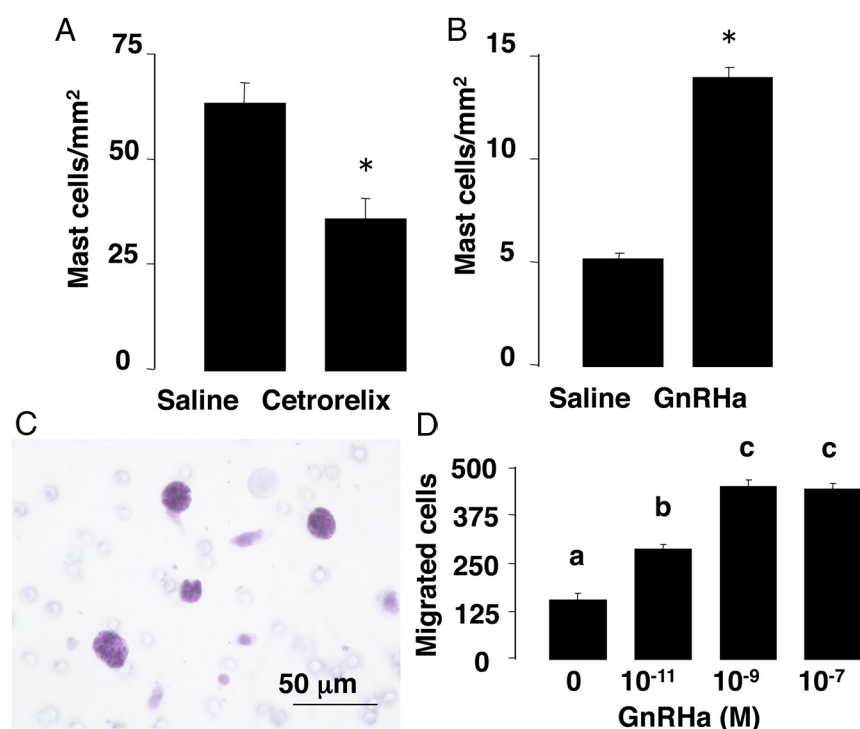


Figure 5. Effects of GnRH on mammary mast cells. A, Cetorelix and saline were locally administered to hemilateral inguinal mammary tissues for 2 days after pups removal on day 10. Then, mammary tissues were collected 2 days later and subjected to tissue examination. *, $P < .001$. B, GnRHa and saline were locally given to hemilateral inguinal mammary tissues for 2 days from day 10 of lactation. Mast cell was counted. *, $P < .001$. C and D, Mast cell migration was assessed by Boyden chamber assay. Number of cells those passed through the polycarbonate membrane was counted. Mast cells (5×10^4 cells/500 μ L) placed in the culture inserts were allowed to migrate towards 10^{-11} M, 10^{-9} M, and 10^{-7} M GnRHa or medium alone in each well for 1 hour at 37°C . After a chemotaxis assay, cells on the membrane were stained with toluidine blue. An example of the stained membrane is shown (C). Each point represents the mean \pm SEM of 4 separate experiments. Different letters indicate significant differences, $P < .05$ (D).

gland (Figure 4A). The large GnRH-positive cells were demonstrated to be mast cells by comparing serial sections stained with anti-GnRH and toluidine blue (Figure 4B). The number of mast cell was increased significantly after weaning on day 21 (Figure 4, C and D), and this coincided with an augmentation in tissue GnRH concentration (Figure 3B). Mast cells and GnRH mRNA expression were also increased 2 days after pup removal during midlactation on day 10 (Figure 4, E and F).

Mast cells were counted in the mammary tissues of forcibly weaned rats after local administration of Cetorelix. Cetorelix significantly reduced mammary mast cells (Figure 5A). On the other hand, locally administered GnRHa increased mammary mast cells even though pups remained (Figure 5B). Because the number of tissue mast cells increased in parallel with the augmentation of tissue GnRH concentration and local administration of GnRH increased mast cell number, we examined whether GnRH could be a chemoattractant for mast cells. Various concentrations of GnRHa were applied to the bottom of Boyden chamber and peritoneal mast cells were placed in the

upper chamber. The number of cells that migrated through the 8- μ m pores during 1 hour of incubation was counted after toluidine blue staining (Figure 5C). GnRHa facilitated the migration of mast cells in a dose-dependent manner (Figure 5D).

PRL and GnRH expression

We examined the relationship between GnRH and the major mammotropin, PRL. Exogenous ovine PRL (30 IU/0.2 mL, every 8 h) suppressed both the expression of ANXA5 and decreased TUNEL-positive cells (Figure 6A) when administered sc every 8 hours for 2 days after pup removal on lactation day 10. PRL also reduced the mRNA expression of ANXA5 and GnRH (Figure 6, B and C) and reduced the number of mast cells (Figure 6D). Inhibition of endogenous PRL with a dopamine agonist (ergocryptine, 150 μ g/100 μ L, ip, once a day from d 10 to 12 of lactation) significantly increased the number of mammary mast cells even though the pups remained (Figure 6E). Consequently, it is concluded that the reduction of plasma PRL caused by weaning is

likely the trigger for GnRH production, mast cell recruitment and involution.

Discussion

We have reported previously that ANXA5 expression in mammary epithelial cells was dramatically increased after weaning (18). The lactation period of rats is set to 21 days in our colony and pups are separated from mother on day 21. After weaning, the mammary gland increases or maintains its weight for a while due to accumulation of milk in the alveoli and then shrinks rapidly. Shrinkage (involution) of mammary tissues is caused by massive apoptosis and clearance of mammary epithelial cells. Because we already found that the expression of ANXA5 is stimulated by GnRH in gonadotropes, luteal cells and Leydig cells (11, 12, 15, 16, 28), we predicted that the increase of ANXA5 intensity in the mammary tissues after weaning

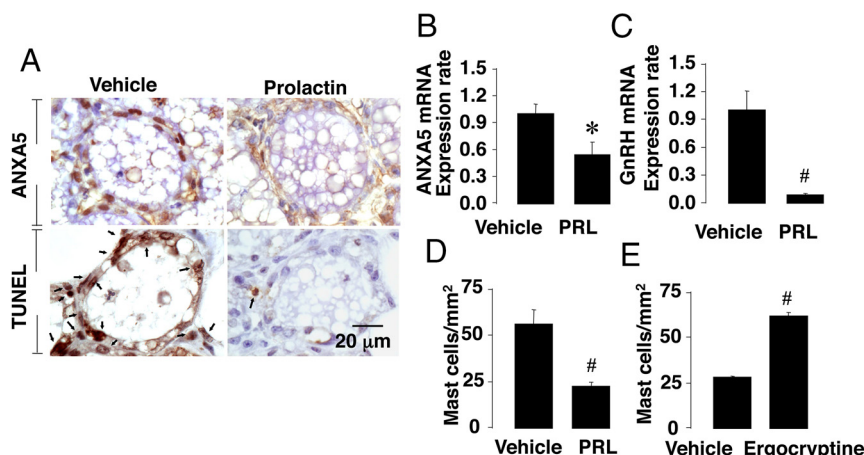


Figure 6. Effects of PRL on mammary GnRH and mast cells. A, The effect of repetitive PRL administration to weaned dams on ANXA5 expression and apoptosis was examined. On day 10 of lactation, pups were removed and 30 IU/200 μ L of PRL was administered every 8 hours for 2 days. B and C, Changes in the mRNA expression of ANXA5 (B) and GnRH (C) mRNA in the mammary gland after PRL administration were evaluated. Effects of PRL on mast cell number were examined. D, PRL was administered to lactating rats from day 10 to 12. Mammary tissues were collected on day 12 and subjected to mast cell counting. E, The number of mammary mast cells was counted in lactating rats after treatment with ergocryptine (150 μ g/100 μ L/d, from d 10 to 12 of lactating rats). Statistical significance was shown with *, $P < .05$ and #, $P < .001$.

would be an effect of GnRH and that GnRH would be involved in the process of mammary involution.

We show here that Cetrorelix suppresses the postlactational increase of ANXA5 expression and also the number of TUNEL-positive apoptotic cells when compared with saline-treated mammary tissues in the same animal. Conversely, local administration of a GnRHa to the inguinal mammary gland of lactating rats increased both ANXA5 expression and TUNEL-positive cells compared with the saline administered side. These data indicate that apoptosis of mammary epithelial cells is induced by locally synthesized GnRH in the tissue. The same experimental protocol showed that the local administration of Cetrorelix after forcible weaning suppressed both ANXA5 and GnRH mRNA expression. In contrast, GnRHa increased both ANXA5 and GnRH mRNA expression in the mammary tissues of lactating rats. We further demonstrated that GnRH action on the mammary gland is physiological. The mammary gland content of GnRH increased after weaning. GnRHa reduced mammary weight, milk production, and casein accumulation in the mammary gland

of lactating rats. These observations suggest an endogenous regulating mechanism of mammary epithelial cells by GnRH.

It has been already demonstrated that mast cells have a role in mammary involution. Lilla et al demonstrated that plasma kallikrein of mast cells is involved in stromal remodeling in postlactational involution (20). Brain mast cells have been reported to express GnRH (24). Here, we showed mammary mast cells were intensely immunostained by GnRH antibodies. Because levels of GnRH in the general circulation are so low, it is assumed that mammary mast cells also synthesize GnRH. However, because some background staining was observed, whether they are the only producers remains to be more rigorously examined.

The number of mast cells in the mammary tissue was increased after the reduction of plasma PRL after normal and premature weaning. Recruitment of mast cells is impaired by exogenous PRL administration and enhanced by a dopamine agonist. The increase of mast cell number correlates very well with the augmentation of GnRH mRNA and the peptide expression. GnRH may be a heretofore unrecognized general mast cell secretory product. We also showed that GnRH is a chemoattractant for mast cells. This suggests a positive feedback loop in which the fall in PRL after weaning causes GnRH production and the initial recruitment of mast cells, then further production of GnRH by the mast cell recruits more mast cells that trigger the massive tissue remodeling during involution, for example through plasma kallikrein (20).

Inhibition of PRL secretion increased mammary mast cells. So, the cessation of PRL release by removal of suckling stimuli is necessary for GnRH synthesis and mast cell infiltration. Because receptors for PRL have been identified on mast cells as well as mammary epithelial cells (29),

Table 1. Antibody Table

Peptide/Protein Target	Antigen Sequence (if Known)	Name of Antibody	Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody	Species Raised in; Monoclonal or Polyclonal	Dilution Used
Rat ANXA5	NP_037264	MS-2	Homemade	Rabbit	1:10 000
GnRH	N.A.	N.A.	Homemade	Rabbit	1:10 000
Rabbit γ -globulin	N.A.	N.A.	Homemade	Goat	N.A.
β -Actin	N.A.	C4	Santa Cruz Biotechnology, Inc	Mouse	1:2000
β -Casein	N.A.	FL-231	Santa Cruz Biotechnology, Inc	Rabbit	1:2000

PRL could exert its suppressive effect on mast cell infiltration and GnRH production directly. However, it was recently demonstrated that PRL mediates kisspeptin neuron to suppress GnRH secretion at hypothalamus (30). So, it may be necessary whether the action of PRL is direct on GnRH producing cells also in the mammary tissues. The present observation is analogous to our previous result that PRL inhibited the ability of GnRH to induce apoptosis of luteal cells in the ovary of pseudopregnant rats (15).

Involution of the mammary gland consists of controlled apoptosis and tissue remodeling. Accumulation of milk in the alveoli increases cytokines and they in turn activate Stat3. Stat3 binds directly to the p55 α and p50 α promoter and the overexpression of p55 α and p50 α suppresses the phosphoinositide 3-kinase-protein kinase B signaling (3). Suppression of protein kinase B results in the activation of the proapoptotic factor bcl-2-like protein 4 that stimulates the executor molecule of apoptosis, Caspase 3 (31, 32). Among cytokines that are involved in the activation of signal transducer and activator of transcription 3, leukemia inhibitory factor is postulated to trigger this sequence of events (5). Because conditional knockout of signal transducer and activator of transcription 3 suppressed mast cell accumulation in the mammary tissue (19), GnRH is hypothesized to work like leukemia inhibitory factor at a very early stage of postlactational involution.

These results are the first to demonstrate that GnRH, synthesized locally in the mammary tissues, is required for mammary involution after lactation. Cessation of PRL secretion triggers GnRH and ANXA5 synthesis in mammary epithelial cells. GnRH induces the infiltration of mast cells into mammary tissue. PRL suppression of GnRH production and hence mast cell infiltration in the mammary tissues is a novel mammotropic function of PRL.

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